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*Full Length Research Paper*

# Studying arsenic trioxide-induced apoptosis of Colo-16 cells with two-photon and confocal laser scanning microscopy

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With two-photon and confocal laser scanning microscopy in combination with fluorescent probes Hoechst 33342, 2',7'-dichlorofluorescein diacetate (DCFH-DA) and Fluo 3-AM, we simultaneously observed arsenic trioxide (As<sub>2</sub>O<sub>3</sub>)-induced changes in nuclear morphology, reactive oxygen species (ROS) and intracellular calcium concentration [Ca<sup>2+</sup>]<sub>i</sub> within human skin squamous carcinoma cells (Colo-16 cells). Our results indicated that As<sub>2</sub>O<sub>3</sub> induced [Ca<sup>2+</sup>]<sub>i</sub> elevation and ROS production within Colo-16 cells, and both [Ca<sup>2+</sup>]<sub>i</sub> elevation and ROS production were involved in the apoptosis of Colo-16 cells. These results suggested that two-photon and confocal laser scanning microscopy might provide a real-time, sensitive and noninvasive method for simultaneously multi-parameter observation of As<sub>2</sub>O<sub>3</sub>-induced apoptosis at the single cell level.

**Key words:** Two-photon laser scanning microscopy, confocal laser scanning microscopy, human skin squamous carcinoma cells (Colo-16 cells), arsenic trioxide, apoptosis.

## INTRODUCTION

Although arsenic is poisonous and chronic arsenic exposure from industrial or natural sources can cause serious toxicity, arsenic has been used therapeutically for more than 2,400 years (Klaassen, 1996); in traditional Chinese medicine, arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) is used to treat syphilis, rheumatosis, and psoriasis (Shen et al., 1997). Recent researches demonstrated that As<sub>2</sub>O<sub>3</sub> induces partial cytodifferentiation and triggers apoptosis of the leukemia cells, leading to high clinical and molecular remission rates in patients with relapsed acute et al., 2001; Zhu et al., 1997) and As<sub>2</sub>O<sub>3</sub> have been approved by Food and Drug Administration of U.S.A. for

promyelocytic leukemia (APL) (Soignet et al., 1998; Muto et al., 2001 use in the treatment of relapsed/refractory APL.

Fluorescence microscopy is an essential tool of modern biology and has emerged as a novel and noninvasive method for visualization of living cells and biological tissue (Gustafsson, 1999). The effective sensitivity of fluorescence microscopy measurements is often limited by out-of-focus flare. This limitation is greatly reduced in a confocal laser scanning microscopy, where the out-of-focus background is rejected by a confocal pinhole to produce thin (< 1 μm) and unblurred optical sections from within thick samples (White et al., 1987). The two-photon laser scanning microscopy is a new alternative to confocal microscopy and is also a promising technique to observe biological specimens because of its inherent advantage such as three-dimensional resolution without a

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confocal pinhole, less photobleaching above and below an observing plane and long depth penetration with near-infrared light excitation (Denk et al., 1990, 1995). In two-photon laser scanning microscopy, the fluorophore is excited by a focus beam of light of approximately twice the wavelength of the normal excitation light. For example, a fluorophore that normally absorbs ultraviolet light (-350 nm) can be excited by two red photons (-700 nm) if they reach the fluorophore at the same time ( $-10^{-18}$  seconds). Therefore, two-photon laser scanning microscopy in combination with confocal laser scanning microscopy might provide the possibility for simultaneously measurement of multi-labeling biological specimens based on the difference between excitation or emission wavelengths of the fluorescent probes.

Although some Chinese medicines used to cure skin cancer contain  $\text{As}_2\text{O}_3$  and show good therapeutic efficacy (Gilman et al., 1996), its accurate mechanism is still unknown. In this paper, two-photon and confocal laser scanning microscopy were used to study  $\text{As}_2\text{O}_3$ -induced changes in nuclear morphology, reactive oxygen species (ROS) and intracellular calcium concentration  $[\text{Ca}^{2+}]_i$  within human skin squamous carcinoma cells (Colo-16 cells). Our results indicated that  $\text{As}_2\text{O}_3$  induced concentration-dependent apoptosis of Colo-16 cells and both  $\text{As}_2\text{O}_3$ -induced  $[\text{Ca}^{2+}]_i$  elevation and ROS production involved in the apoptosis of Colo-16 cells, suggesting that two-photon and confocal laser scanning microscopy might throw new insight into the anti-skin-cancer mechanism of  $\text{As}_2\text{O}_3$ .

## MATERIALS AND METHODS

$\text{As}_2\text{O}_3$  was purchased from Sigma (St Louis, Missouri, U.S.A). 1 mM stock solution (in RPMI 1640 medium at 0 to 4°C storage) was prepared and diluted to a working concentration before use. RPMI 1640 and foetal calf serum were bought from GIBCO-BRL (Grand Island, NY, USA). Fluorescent probes Hoechst 33342, 2',7'-dichlorofluorescein diacetate (DCFH-DA) and Fluo 3-AM were purchased from Molecular Probes Inc. (Eugene, Oregon, U.S.A.). Trypsin, ethylenediaminetetra-acetate (EDTA), sodium dodecyl sulphate (SDS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma.

### Cell culture

The human skin squamous carcinoma cell lines (Colo-16 cells) were provided by the Department of Biology, Beijing Normal University. All cells were cultured in RPMI 1640 containing 10% fetal bovine serum in an incubator containing 5%  $\text{CO}_2$ . Exponentially growing cells were used in all the experiments.

### Cell viability assays

100  $\mu\text{l}$  of cell suspension ( $1 \times 10^4$  Colo-16 cells) was seeded into 96-well tissue culture plates containing different-concentration of  $\text{As}_2\text{O}_3$ . After treatment for 12, 24, 36, 48 and 60 h separately, 10  $\mu\text{l}$  of MTT [5 mg/ml in phosphate-buffered saline (PBS)] was added into each well. After 4 h of incubation at 37°C, the formazan crystals

produced in viable cells were dissolved with 100  $\mu\text{l}$  of 10% SDS.  $\text{OD}_{540}$  was measured on a spectrophotometer to determine the relative cell viability.

### Detection of DNA fragmentation

Colo-16 cells at a density of  $10^6$  cells/ml were treated with different-concentration of  $\text{As}_2\text{O}_3$  for 24 h and then were collected by centrifugation at 2500  $g$  for 5 min. The resultant cell pellets were resuspended in phosphate-buffered saline (PBS) buffer containing 5 mM  $\text{MgCl}_2$  and lysed in 500  $\mu\text{l}$  of TE buffer containing 0.1% SDS and 1.5 mg/ml proteinase K overnight at 37°C. After two successive extractions with phenol-chloroform, the aqueous layer was transferred to a new centrifuge tube. The DNA was precipitated with ethanol, resuspended in 100  $\mu\text{l}$  water and treated with 400  $\mu\text{g}$ /ml RNase A for 2 h at 37°C. Electrophoresis was performed in 1% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). After electrophoresis, the gel was stained with 1 mg/ml ethidium bromide, watched and photographed under UV light.

### Nuclear morphological observation with two-photon laser scanning microscopy

Following treatment with 0, 4 and 10  $\text{As}_2\text{O}_3$  separately for 24 h, cells were loaded with 10  $\mu\text{g}$ /ml Hoechst 33342 in RPMI 1640 for 20 min at 37°C. After rinsing three times with modified Hanks' balanced salt solution (HBSS; in mM: 137 NaCl, 5.37 KCl, 0.81  $\text{MgSO}_4$ , 0.44  $\text{KH}_2\text{PO}_4$ , 0.37  $\text{Na}_2\text{HPO}_4$ , 10 HEPES (pH 7.2), 5.56 glucose and 1.37  $\text{CaCl}_2$ ), cells were imaged with a Bio-Rad MRC 1024MP two-photon laser scanning microscopy at 730 nm excitation and >460 nm emission by using a long-pass emission filter.

### Measurement of $[\text{Ca}^{2+}]_i$ with confocal laser scanning microscopy

Cells were preloaded with 5  $\mu\text{M}$  Fluo 3-AM in RPMI 1640 for 1 h at 37°C. After washing with HBSS three times, cells were treated with 10  $\mu\text{M}$   $\text{As}_2\text{O}_3$  and images of  $[\text{Ca}^{2+}]_i$  were recorded every 20 s for up to 10 min with confocal laser scanning microscopy at 488 nm excitation and 525 nm emission.

### Measurement of reactive oxygen species (ROS) with confocal laser scanning microscopy

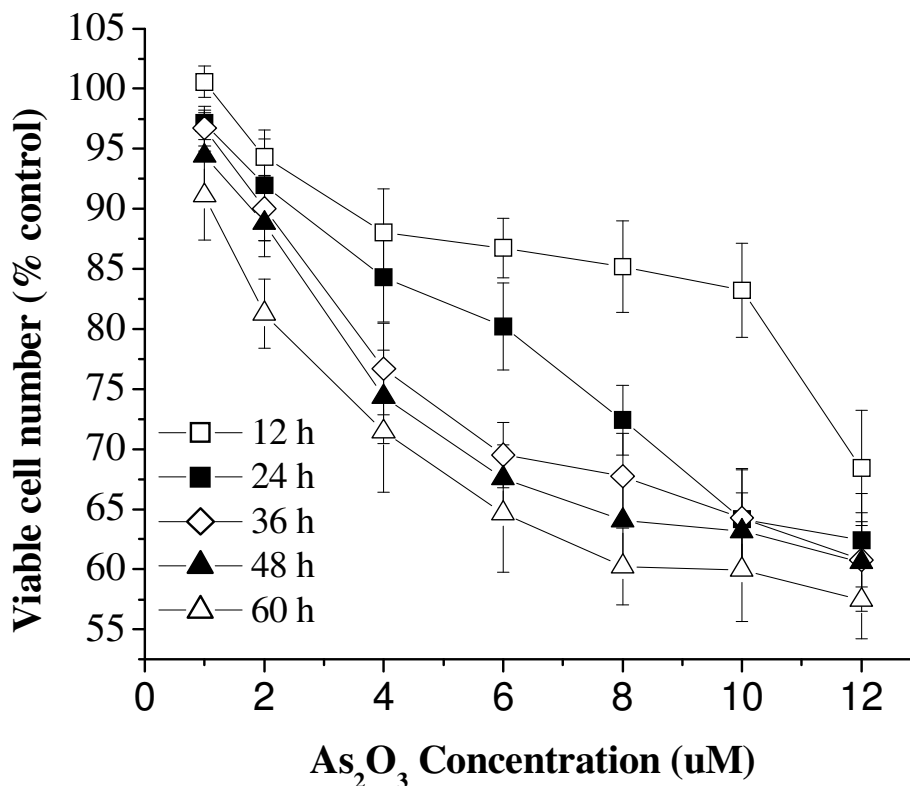
Cells were loaded with 20  $\mu\text{M}$   $\text{H}_2\text{DCF-DA}$  in a HEPES-buffered salt solution (HBSS; in mM: 137 NaCl, 5.37 KCl, 0.81  $\text{MgSO}_4$ , 0.44  $\text{KH}_2\text{PO}_4$ , 0.37  $\text{Na}_2\text{HPO}_4$ , 10 HEPES (pH 7.2) and 5.56 glucose, 1.37  $\text{CaCl}_2$ ) for 15 min at 37°C. After rinsing with HBSS twice, cells were imaged with a Bio-Rad MRC 1024MP confocal laser scanning microscope at 488 nm excitation and 510 nm emission.

Fluorescence from one field of cells per cover slip was typically recorded under different experimental conditions. After acquiring the images of baseline fluorescence levels, 10  $\mu\text{M}$   $\text{As}_2\text{O}_3$  was applied and 6 additional images were obtained every 5 min.

## RESULTS

### Cell viability assays

When Colo-16 cells were cultured for 24 to 60 h with



**Figure 1.** Cytotoxic effect of As<sub>2</sub>O<sub>3</sub> on Colo-16 cells. Viable cell number was determined by MTT assay as described in materials and methods. Data shown are expressed as percentage of control values (without As<sub>2</sub>O<sub>3</sub> treatment) and represented by the mean  $\pm$  SEM (n = 5).

As<sub>2</sub>O<sub>3</sub> (1 to 12  $\mu$ M), cell viability were analyzed by MTT and the viable cell number was compared with the untreated control. Figure 1 shows that the viable cell number decreased with both treatment-time and As<sub>2</sub>O<sub>3</sub>-concentration increased suggesting that the cytotoxic effect of As<sub>2</sub>O<sub>3</sub> on Colo-16 cells was both time and concentration dependent.

#### Detection of DNA fragmentation with electrophoresis and nuclear morphological observation with two-photon laser scanning microscopy

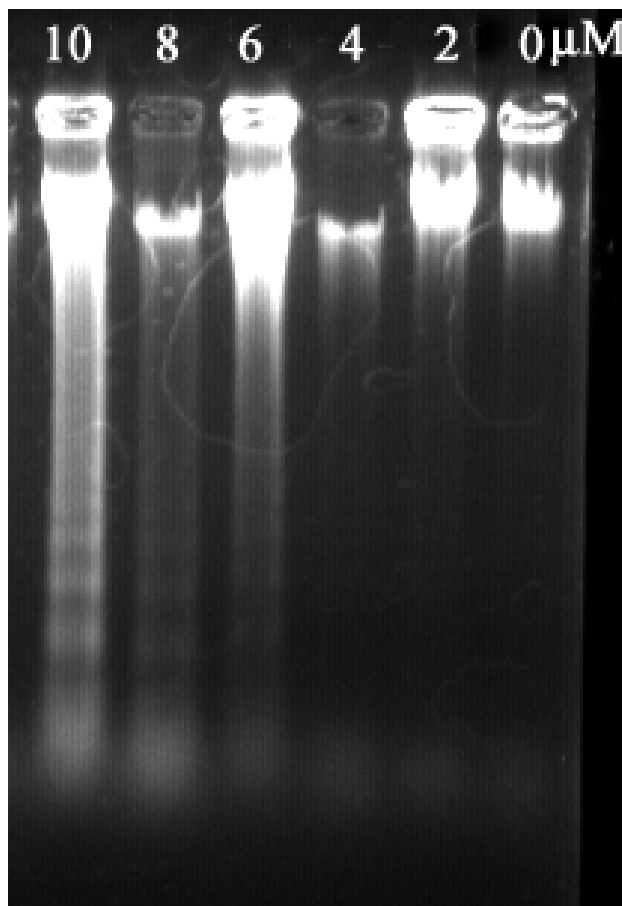
Cells die either following necrosis or apoptosis. Apoptosis (programmed cell death) is an important feature of normal development as well as various disease states, particularly cancer (Kerr et al., 1994). Apoptosis is distinguished from necrosis by characteristic morphological and biochemical changes, including compaction and fragmentation of the chromatin, cell shrinkage and fragmentation into membrane-bound apoptotic bodies, the activation of cysteine proteases such as caspases and internucleosomal fragmentation of DNA by selectively activated DNases (Kerr et al., 1994).

To assess whether Colo-16 cells underwent apoptosis

after exposure up to 10  $\mu$ M As<sub>2</sub>O<sub>3</sub>, we examined the DNA nucleosomal fragmentation via agarose gel electrophoresis (Figure 2) and nuclear morphology via two-photon laser scanning microscopy (Figure 3).

The biochemical hallmark of apoptosis is the degradation of DNA by endogenous DNases, which cut the internucleosomal regions into double-stranded DNA fragments of 180 to 200 bp (characteristic DNA ladder in gel electrophoresis) (Kerr et al., 1994). The results (Figure 2) showed that no DNA ladder appeared with As<sub>2</sub>O<sub>3</sub> concentration of less than 8  $\mu$ M, a faint DNA ladder emerged with 8  $\mu$ M As<sub>2</sub>O<sub>3</sub> and distinct DNA ladder appeared with 10  $\mu$ M As<sub>2</sub>O<sub>3</sub>. The result demonstrated that As<sub>2</sub>O<sub>3</sub> induced the apoptosis of Colo-16 cells in a concentration-dependent manner.

As shown in Figure 3, the nuclear morphological observation showed that all the examined cells without As<sub>2</sub>O<sub>3</sub> treatment had the normal nuclear morphology (Figure 3a). After treatment with 4  $\mu$ M As<sub>2</sub>O<sub>3</sub> for 24 h, some of the examined cells had characteristic chromatin condensation and apoptotic body formation (Figure 3b). Figure 3c shows that after treatment with 10  $\mu$ M As<sub>2</sub>O<sub>3</sub> for 24 h, most of the examined cells had apoptotic body formation. These results further confirmed that As<sub>2</sub>O<sub>3</sub> induced apoptosis of Colo-16 cells in a concentration-



**Figure 2.** DNA electrophoresis of Colo-16 cells after treatment with  $\text{As}_2\text{O}_3$  of different concentration for 24 h.

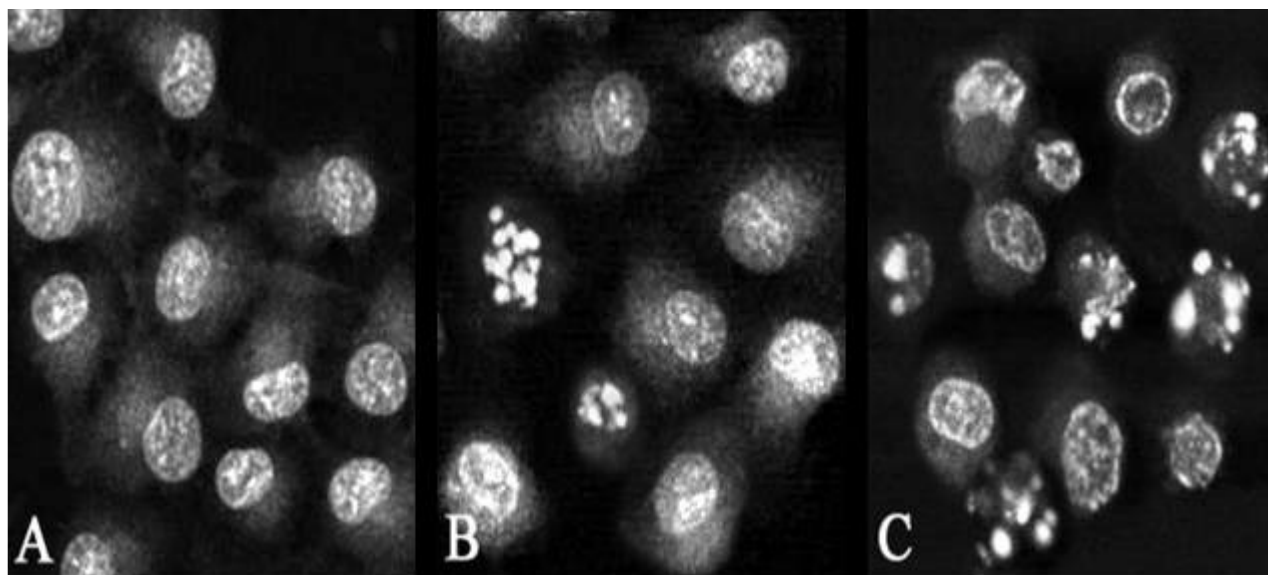
dependent manner.

#### **Measurement of $[\text{Ca}^{2+}]_i$ with confocal laser scanning microscopy**

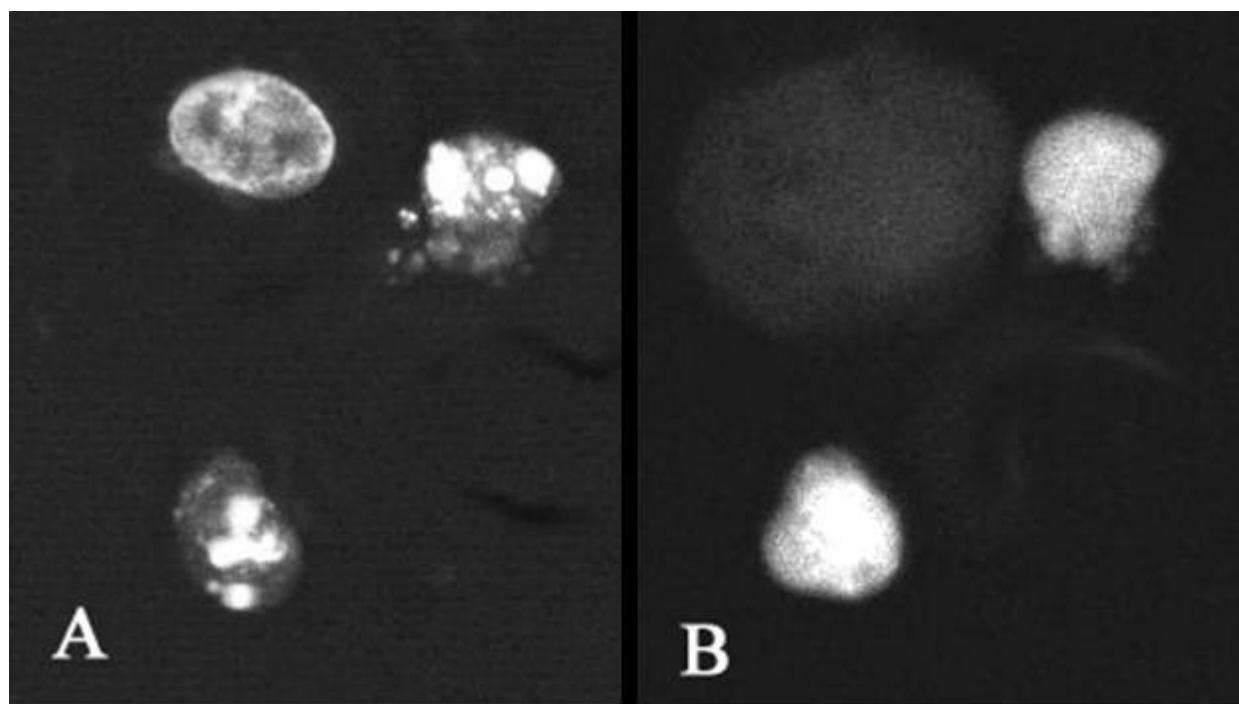
It had been demonstrated that increase in  $[\text{Ca}^{2+}]_i$  played an important role in the induction of  $\text{Ca}^{2+}$ -dependent endonucleases and in initiation of apoptosis (McConkey et al., 1989, 1988). Simultaneously, observation of nuclear morphological changes via two-photon laser scanning microscopy and  $[\text{Ca}^{2+}]_i$  changes via confocal laser scanning microscopy revealed that the apoptotic cells showed high Fluo-3 fluorescence after 10  $\mu\text{M}$   $\text{As}_2\text{O}_3$  treatment for 24 h (Figure 4), and this indicated  $[\text{Ca}^{2+}]_i$  elevation in the apoptotic cells. To confirm whether the  $[\text{Ca}^{2+}]_i$  elevation was induced by  $\text{As}_2\text{O}_3$  stimulation, we studied  $\text{As}_2\text{O}_3$ -induced changes in  $[\text{Ca}^{2+}]_i$  in a real time with confocal laser scanning microscopy. The results showed that  $\text{As}_2\text{O}_3$  rapidly increased  $[\text{Ca}^{2+}]_i$  over the treatment time (Figure 5). Both Figures 4 and 5 suggested that  $\text{As}_2\text{O}_3$ -induced  $[\text{Ca}^{2+}]_i$  elevation was involved in the apoptosis of Colo-16 cells.

#### **Measurement of reactive oxygen species (ROS) with confocal laser scanning microscopy**

Accumulating evidence pointed to ROS as being second messengers in the activation of enzymes, transcription factors, growth, differentiation and apoptosis (Suzuki et al., 1997; Finkel, 1998; Zhang et al., 2001). In this study, fluorescent probe  $\text{H}_2\text{DCF-DA}$  was used to study  $\text{As}_2\text{O}_3$ -induced ROS production.  $\text{H}_2\text{DCF-DA}$  was not sensitive to the oxidizing agents; when  $\text{H}_2\text{DCF-DA}$  was added to the cells, it diffused across the cell membrane and was hydrolyzed by intracellular esterases to  $\text{H}_2\text{DCF}$ ; upon oxidation,  $\text{H}_2\text{DCF}$  yielded highly fluorescent  $\text{DCF}$  (Zhu et al., 1994). To assess whether ROS was involved in the  $\text{As}_2\text{O}_3$ -induced apoptosis of Colo-16 cells, we simultaneously observed the nuclear morphological changes via two-photon laser scanning microscopy and ROS production via confocal laser scanning microscopy. The results showed that  $\text{DCF}$  fluorescence increased in the apoptotic cells with chromatin condensation and apoptotic body formation (Figure 6), indicating ROS production in the apoptotic cells. To confirm whether the ROS formation was induced by  $\text{As}_2\text{O}_3$  stimulation, we



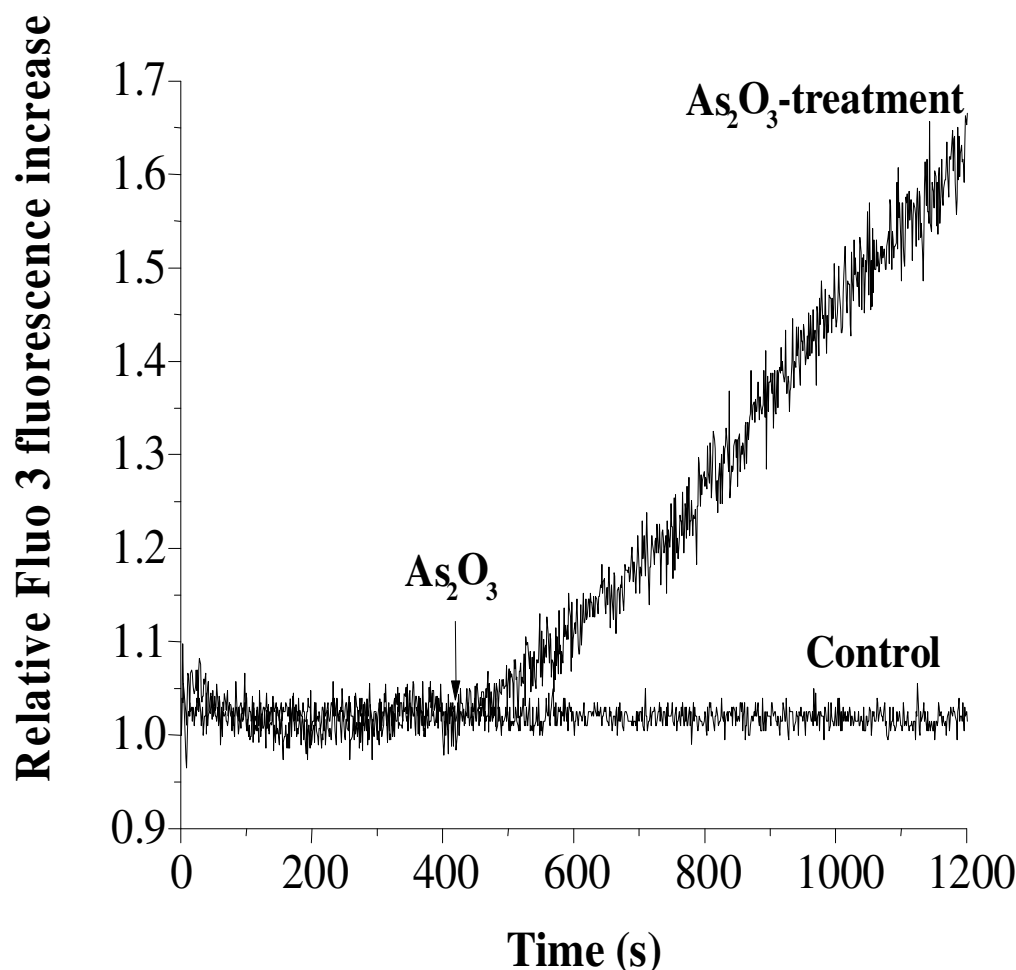
**Figure 3.** Nuclear morphological changes in Colo-16 cells after treatment with  $\text{As}_2\text{O}_3$  of different concentration for 24 h. A, 0  $\mu\text{M}$   $\text{As}_2\text{O}_3$ ; B, 4  $\mu\text{M}$   $\text{As}_2\text{O}_3$ ; C, 10  $\mu\text{M}$   $\text{As}_2\text{O}_3$ . Images of Hoechst 33342 fluorescence was collected by two-photon laser scanning microscopy at 730 nm excitation and  $>460$  nm emission.



**Figure 4.**  $\text{As}_2\text{O}_3$  induced nuclear morphological changes (A) and intracellular calcium changes (B) in Colo-16 cells. Images of Hoechst 33342 fluorescence (A) and Fluo 3 fluorescence (B) were collected simultaneously by two-photon laser scanning microscopy and confocal laser scanning microscopy.

quantitatively investigated  $\text{As}_2\text{O}_3$ -induced ROS production in a real time with confocal laser scanning microscopy. The results showed that the DCF fluorescence increased

greatly over time in response to  $\text{As}_2\text{O}_3$  stimulation; however, DCF fluorescence underwent no dramatic change in the control experiments in which  $\text{H}_2\text{DCF-DA}$



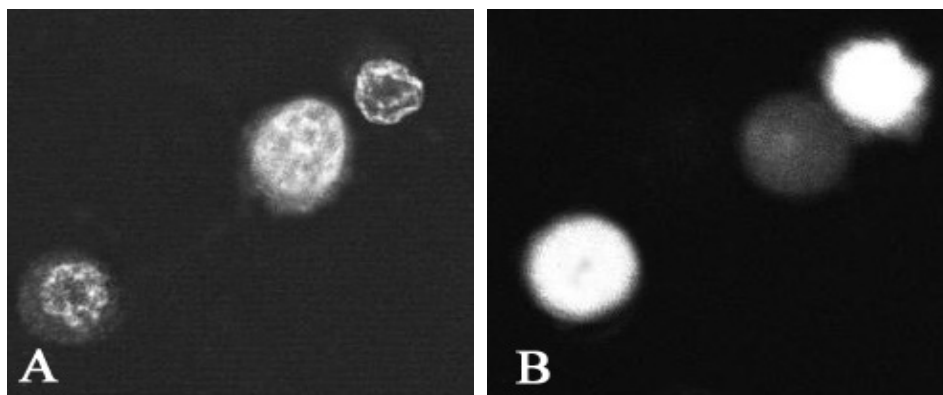
**Figure 5.**  $\text{As}_2\text{O}_3$  induced  $[\text{Ca}^{2+}]_i$  elevation within Colo-16 cells. Images of  $[\text{Ca}^{2+}]_i$  were recorded every 10 s for up to 10 min. with confocal microscopy.  $[\text{Ca}^{2+}]_i$  is expressed as emission intensity of Fluo 3 at 525 nm. The PRESENTED data was recorded for a representative single cell and was presented as increase in Fluo 3 fluorescence over time, in multiples of fluorescence at time 0 (= 1).

was loaded without  $\text{As}_2\text{O}_3$  addition (Figure 7). Both Figures 6 and 7 suggested that  $\text{As}_2\text{O}_3$ -induced ROS was involved in the apoptosis of Colo-16 cells.

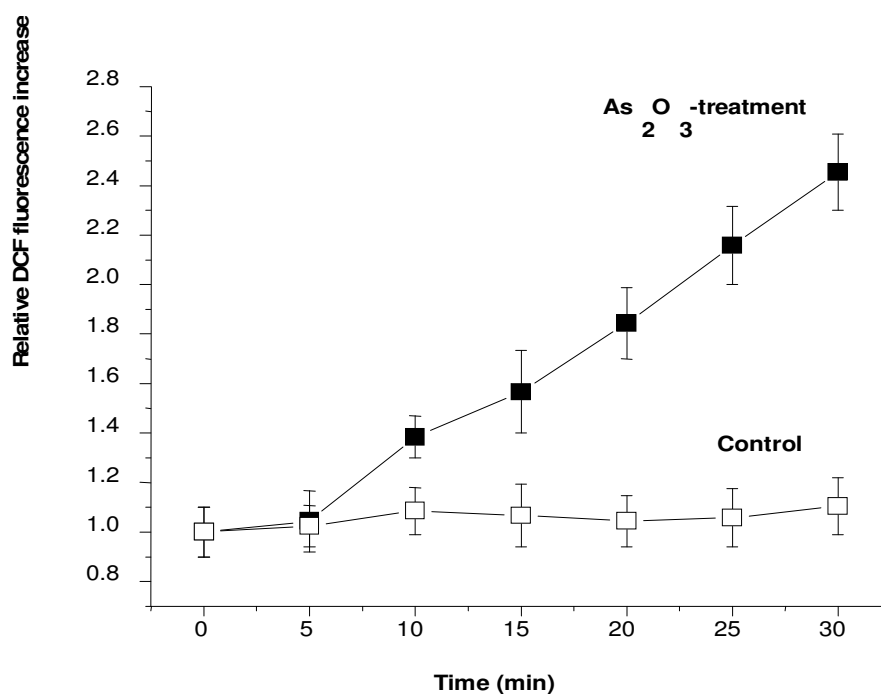
## DISCUSSION

Arsenic trioxide ( $\text{As}_2\text{O}_3$ ) has been widely used in the clinic treatment of relapsed/refractory acute promyelocytic leukemia (APL); however, exactly how  $\text{As}_2\text{O}_3$  mediates its clinical efficacy is not fully understood. Two main mechanisms of action of  $\text{As}_2\text{O}_3$  have been identified from both *in vivo* and *in vitro* studies: promotion of APL cell differentiation (observed at low levels of  $\text{As}_2\text{O}_3$ ) and induction of apoptosis (observed at high levels of  $\text{As}_2\text{O}_3$ ) (Chen et al., 1997; Shao et al., 1998). The therapeutic potential of  $\text{As}_2\text{O}_3$  might be extended to malignant

diseases beyond APL.  $\text{As}_2\text{O}_3$  has been reported to have direct cytotoxic effects on different cancer cell lines including leukemic, lymphoma and a variety of solid tumor cell lines in high concentrations (Lee and Ho, 1994). This study showed that  $\text{As}_2\text{O}_3$  had direct cytotoxic effect on human skin squamous carcinoma cells (Colo-16 cells) in both concentration- and time-dependent manner (Figure 1). Further study with both agarose gel electrophoresis (Figure 2) and two-photon laser scanning microscopy (Figure 3) demonstrated that  $\text{As}_2\text{O}_3$  induced apoptosis of Colo-16 cells at higher concentration ( $> 8 \mu\text{M}$ ), suggesting that apoptosis appeared to be the main phenomenon resulting in significant cell death at higher concentrations of  $\text{As}_2\text{O}_3$ .  $\text{As}_2\text{O}_3$ -induced apoptosis occurs via a variety of mechanisms (Miller, 2002). It has been reported that superoxide generation was the main mechanism of apoptosis by  $\text{As}_2\text{O}_3$  in several experimental



**Figure 6.**  $\text{As}_2\text{O}_3$ -induced nuclear morphological changes (A) and ROS production (B) in Colo-16 cells. Images of Hoechst 33342 fluorescence (A) and DCF fluorescence (B) were collected simultaneously by two-photon laser scanning microscopy and confocal laser scanning microscopy.



**Figure 7.**  $\text{As}_2\text{O}_3$ -induced ROS production within Colo-16 cells. ROS production in single cells was expressed as a relative fluorescence increase as compared to the starting fluorescence. The data represents the mean  $\pm$  SEM of at least 80 cells in each condition.

models (lee and Ho, 1994; Lynn et al., 2000; Jing and Dai, 1999). Generation of reactive oxygen species can cause loss of mitochondrial membrane potential with subsequent changes in membrane permeability and arsenic trioxide can further inhibit glutathione peroxidase and increase cellular hydrogen peroxide content (Jing and Dai, 1999). Our study with the two-photon and confocal laser scanning microscopy (Figure 4) demonstrated that ROS production increased in the apoptotic cells with chromatin condensation and

apoptotic body formation and real-time analysis with confocal microscopy (Figure 5) showed that ROS increased greatly over time in response to  $\text{As}_2\text{O}_3$  stimulation, suggesting that  $\text{As}_2\text{O}_3$ -induced ROS was involved in apoptosis of Colo-16 cells. The increase in  $[\text{Ca}^{2+}]_i$  played an important role in the induction of  $\text{Ca}^{2+}$ -dependent endonucleases and in the initiation of apoptosis (McConkey et al., 1989, 1988), although in some cases, apoptosis proceeds in the absence of  $[\text{Ca}^{2+}]_i$  changes (Whyte et al., 1993). It has been reported

that intracellular calcium change was involved in As<sub>2</sub>O<sub>3</sub>-induced apoptosis in three myelocytic leukemia cell lines (Wei et al., 2001). Our study observed with two-photon and confocal laser scanning microscopy (Figure 6) demonstrated that [Ca<sup>2+</sup>]<sub>i</sub> was increased in the apoptotic cells with chromatin condensation and apoptotic body formation. Real-time analysis with confocal microscopy (Figure 7) showed that [Ca<sup>2+</sup>]<sub>i</sub> increased greatly over time in response to As<sub>2</sub>O<sub>3</sub> stimulation, suggesting that As<sub>2</sub>O<sub>3</sub>-induced [Ca<sup>2+</sup>]<sub>i</sub> elevation was involved in the apoptosis of Colo-16 cells.

Apoptosis occurs in many different cell types and in response to diverse stimuli including DNA damage, intracellular damage, toxins and extracellular signals (Kerr et al., 1994). Apoptosis has become a target subject for understanding cellular mechanisms of many diseases, as well as for developing new drugs that interfere with either proapoptotic or antiapoptotic molecular networks. Consequently, it has become important to develop reliable assays to detect apoptosis. Techniques currently available for apoptosis detection are based on the study of morphology of apoptotic cells (light and fluorescence microscopy coupled to nuclear staining with specific dyes and electron microscopy), DNA fragmentation detected by electrophoretic techniques and terminal transferase-mediated dUTP nick-end labeling (TUNEL) and membrane changes detected by annexin V *in vivo* labeling and on immunological assays using antibodies directed to apoptosis-related proteins (Stadelmann and Lassmann, 2000). However, all the earlier mentioned techniques detect only one specific feature of the process of apoptosis. Two-photon and confocal laser scanning microscopies in combination with specific fluorescent dyes provide the possibility for simultaneously multi-parameter observation of apoptosis at the level of single cell. In our study, with combination of two-photon and confocal laser scanning microscopy, we simultaneously observed the changes in nuclear morphology, [Ca<sup>2+</sup>]<sub>i</sub> and ROS production during As<sub>2</sub>O<sub>3</sub>-induced apoptosis of Colo-16 cells, suggesting that two-photon and confocal laser scanning microscopy might provide a real-time, sensitive and noninvasive method for simultaneously multi-parameter observation of drug-induced apoptosis at the single cell level.

## Conclusions

With two-photon and confocal laser scanning microscopy in combination with fluorescent probes Hoechst 33342, DCFH-DA and Fluo 3-AM, we simultaneously observed As<sub>2</sub>O<sub>3</sub>-induced changes in nuclear morphology, ROS and [Ca<sup>2+</sup>]<sub>i</sub> within Colo-16 cells. Our results indicated that both As<sub>2</sub>O<sub>3</sub>-induced [Ca<sup>2+</sup>]<sub>i</sub> elevation and ROS production were involved in the apoptosis of Colo-16 cells. These results suggested that two-photon and confocal laser scanning microscopy might provide a real-time, sensitive and noninvasive method for simultaneously

multi-parameter observation of drug-induced apoptosis at the single cell level.

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